**PATENT**
**GOUD:040US**

# APPLICATION FOR UNITED STATES LETTERS PATENT

**For**

# CYTOKINE RECEPTOR MODULATORS, METHOD OF IDENTIFYING SAME, AND METHOD OF MODULATING CYTOKINE RECEPTORS ACTIVITY WITH SAME

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| EXPRESS MAIL MAILING LABEL | |
|---|---|
| EXPRESS MAILING LABEL NO.: | EV 323287438 US |
| DATE OF DEPOSIT: | October 24, 2003 |

This application claims the benefit of U.S. Provisional Application No. 60/420,679, filed October 24, 2002, and U.S. Provisional Application No. 60/423,530, filed November 5, 2002. The entire text of the above provisional applications are specifically incorporated by reference.

## TITLE OF THE INVENTION

CYTOKINE RECEPTOR MODULATORS, METHOD OF IDENTIFYING SAME, AND METHOD OF MODULATING CYTOKINE RECEPTORS ACTIVITY WITH SAME

## FIELD OF THE INVENTION

The present invention relates to cytokine receptor agonists and antagonists, to a method of identifying same, and to a method of modulating cytokine receptors activity with same. More specifically, the present invention is concerned with extracellular, non-competitive cytokine receptor modulators, a method of identifying same, their identification and their uses. More particularly, the present invention is concerned with extracellular, non-competitive cytokine receptor antagonists, a method of identifying same, their identification and their therapeutic uses.

## BACKGROUND OF THE INVENTION

**[0001]** Cytokines are generic terms for designating biologically active hormone-like proteins (interleukins, interferons, tumor necrosis factor, growth factors) that mediate their effects through a superfamily of receptors. Cytokines and their receptors constitute a powerful control network by which cells signal and coordinate cell proliferation and differentiation, cell death and survival. Cytokines are low molecular weight peptides having very potent

biological activity. Their mechanism of action is generally autocrine and paracrine and act by ultimately regulating gene expression.

[0002] Cytokines and their receptors are thus implicated in major diseases. They regulate hematopoiesis, immunity and development of the nervous system. Most of all, they contribute to the development of afflictions such as cancer, inflammatory and autoimmune reactions, asthma, allergy, thrombosis, vascular diseases and septic shock by influencing aberrant or overexpressing genes leading to diseases. Cytokines and growth factors mediate tightly regulated biological effects in order to ensure proper control and functioning of the immune system. Therefore, cytokines are also involved in pathological conditions such as inflammation (e.g. rhumatoid arthritis) and tissue degeneration. Diseases which may develop or progress as a result of defects in cytokine or growth factor mediated cell signaling have a high prevalence in the population and are associated with significant morbidity and/or mortality. For these reasons cytokine receptors are important therapeutic target.

[0003] The treatments available for these pathologies are currently limited. They often result in high toxicity and secondary effects. The demand in the medical world for safer and more targeted therapies is therefore considerable.

[0004] The current approaches in the field of cytokines antagonists include the development of soluble receptors, monoclonal antibodies directed against cytokines, mimetics of cytokines, antisense techniques and kinases inhibitors. Few of these strategies have been successful in drug development, however. Nevertheless, certain antibodies targeting the ligand and the receptor, natural soluble receptor inhibitors (eg. IL1ra), and decoy soluble receptors have

displayed interesting results. For instance, Trastuzumab (Herceptin, Roche) a monoclonal antibody which binds the HER-2/neu protein tyrosine kinase, and ZD1839 (Iressa, Astra-Zeneca), a small molecule which binds the EGF receptor are either in clinical trials or available for the treatment of certain diseases.

**[0005]** Non competitive antagonists of cytokines have also been described. In international application no. WO 93/14781 published in 1993, Fox describes the use of non-competitive peptides targeting intracellular domains of EGF. Intracellular domains are difficult to reach by peptides because of the barrier that the cell membrane constitutes.

**[0006]** Antagonists of the prior art are thus either competitive (e.g. soluble receptors, antibodies, cytokine mimetics), not very selective (e.g. tyrosine kinase inhibitors), costly to produce or difficult to apply *in vivo* (e.g. antisense). Because the ligand exceeds by far the concentration of the receptor, the concentration of competitive antagonists needed to inhibit the receptor is often substantial.

**[0007]** There is therefore a need for non-competitive, selective, extracellular and simple to identify, select and produce antagonists of cytokines.

**[0008]** The present invention seeks to meet these needs and other needs.

**[0009]** The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

## SUMMARY OF THE INVENTION

**[0010]** The present invention thus concerns non-competitive and selective extracellular cytokine receptor modulators, and methods of selecting and of using same.

**[0011]** The peptides, derivatives and peptidomimetics thereof of the present invention are derived from selected cytokine receptor flexible regions. Cytokine receptor agonists or antagonists of the present invention possess a unique mechanism and site of action for inhibiting cytokine receptors activity. They are peptides strategically positioned on at least one of an extracellular flexible region including juxtamembranous regions, flexible regions between domains of the cytokine receptor, and oligomerization site, that are important for the appropriate conformation of the receptor which enables signaling. In one embodiment the flexible region is required for proper oligomerization to occur. In such an embodiment, appropriate conformation of the receptor is needed to allow adequate positioning of the protein chains to enable oligomerization of the receptor and its resulting activation.

**[0012]** Cytokine receptors subfragments or peptides of this invention may promote or stabilize a particular conformation of the cytokine receptor which results in inhibition or activation of the receptor activity. In particular, the antagonists of this invention do not necessarily interfere directly with the oligomerization site. They may, for example, exert their antagonistic activity by directly or indirectly preventing the oligomerization of the complementary protein chains (of homodimers as well as heterodimers receptors) of the extracellular domain of the cytokine receptor. This process effectively prevents activation of the intracellular receptor domains responsible for cytokine enzymatic function. Subsequent cell transduction events leading to

overexpression of the ligand and/or cell bound receptors responsible in part for disease expression are thereby prevented.

**[0013]** In the alternative, one can use cytokine receptors subfragment peptides or derivatives to promote or stabilize the active cytokine receptor structure capable signal transduction. Such peptides are considered agonists of the present invention. Cytokine receptor modulators of the present invention possess a number of advantages over the prior art.

**[0014]** Because they have extracellular targets, unlike certain known drug candidates which target intracellular regions of the cytokine receptors, the antagonists of the present invention do not necessitate a prior permeabilization or other disruption of cell membranes to gain access to the target in order to produce a pharmacological response.

**[0015]** Because they are non competitive, a smaller amount of the antagonists of the present invention is necessary to inhibit the receptor that they target, as compared to competitive inhibitors.

**[0016]** As peptides, the antagonists of the present invention are advantageously simple to synthesize.

**[0017]** In order to provide a clear and consistent understanding of terms used in the present description, a number of definitions are provided herein below.

**[0018]** In view of the importance of the function of cytokine receptors in numerous pathway and conditions in animals, the present invention has broad

impact on the screening, identification, validation and treatment of conditions or diseases associated with abnormal functioning of these cytokine receptors.

**[0019]** Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).

<u>Cytokine receptors</u>

**[0020]** The term "cytokine" refers herein to any cytokine including growth factor. Similarly, the term "cytokine receptors" refers herein to any cytokine receptor including growth factor receptors. The cytokine receptors comprise a number of families including 1- tyrosine kinases receptors, such as vascular endothelial growth factor receptors (VEGFR), PDGFR, IGF-1R, FGFR, EGFR; 2- type I receptors, such as Interleukins-2, 3, 4, 5, 7, 9 and 15; 3- type II receptors, such as interleukins 10, IFN$\alpha$R, IFN$\beta$R, IFNR,; 4- TGF$\beta$; 5- chemokines; and 6- NGF/TNF; 6- interleukins-1 types I and II. The present invention encompasses peptidic agonists or antagonists directed at any cytokine.

**[0021]** The method of identifying cytokines antagonists of the present invention is based on the localization of flexible extracellular regions, including regions between domains, long loops between two $\beta$ chains, as well as juxtamembranous regions of the receptor, which are important for the

appropriate conformation and/or oligomerization of the subunits of the receptor and/or its resulting activation. These regions can be determined using for example crystallography data, model structures, data bases, sequence alignments and the like. For example, the targeted regions were established herein based on crystal structure data provided by crystallography for IL-1R and IGF-1R and on published model structure for IL-4R. Databases such as Swiss-Prot and NCBI as well as sequences alignments with CLUSTALW and MOTIFSCAN enabled a comparison between many regions constituting the receptors domains and their structural similarities with flexible regions of the vascular endothelial growth factor receptor (VEGFR). It should be noted that the flexible regions of the present invention need not be directly involved in oligomerization. Indeed, regions which facilitate oligomerization or regions that are implicated in conformational changes needed for receptor signaling are also within the scope of the present invention. The same principle apply to the identification of cytokine agonists.

**[0022]** The terminology "juxtamembranous region of a receptor" refers herein to an extracellular region of the receptor located in the vicinity of the cellular membrane. More particularly in a region which spans a length of up to about 20 amino acids.

**[0023]** The terminology "flexible region of a receptor" refers herein to any region of the receptor that possesses sufficient flexibility to enable this region to bend, extend, twist or otherwise change its conformation and by which conformational change alone or in combination with other conformational changes of other flexible regions, receptor's activity is induced or facilitated. It includes juxtamembranous regions, oligomerization regions including those having secondary structures such as $\alpha$ helix, $\beta$ sheet, loops, $\beta$ turns, and flexible regions between domains of the receptor or in long loops between two

$\beta$ chains.

Peptides preparation

**[0024]** The peptides of this invention, including the analogs and other modified variants, may generally be synthesized according to the FMOC protocol in an organic phase with protective groups. They can be purified with a yield of 70% with HPLC on a C18 column and eluted with an acetonitrile gradient of 10-60%. Their molecular weight can then be verified by mass spectrometry.

**[0025]** The peptides of the invention may also be prepared according to the solid phase synthetic method. For example, the solid phase synthesis is well known and is a common method for preparation of peptides, as are a variety of modifications of that technique [Merrifield (1964), J. Am. Chem. Soc., 85: 2149; Stewart and Young (1984), Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford, Ill.; Bodansky and Bodanszky (1984), The Practice of Peptide Synthesis, Springer-Verlag, New York; Atherton and Sheppard (1989), Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, New York].

**[0026]** Alternatively, peptides of this invention may be prepared in recombinant systems using polynucleotide sequences encoding the peptides. It is understood that a peptide of this invention may contain more than one of the above described modifications within the same peptide. Also included in this invention are pharmaceutically acceptable salt complexes of the peptides of this invention or their derivatives.

Peptides

**[0027]** Peptides of the present invention may therefore be constituted

solely of L-amino acid sequences identical to amino acid sequences of flexible regions of an animal cytokine receptor and preferably of the human cytokines receptor that they target (subfragment peptides) and any mutated peptide that can be generated.

**[0028]** While subfragment peptides are effective in inhibiting wild-type cytokines *in vitro*, their effectiveness *in vivo* might be compromised by the presence of proteases. Serum proteases have specific substrate requirements. The substrate must have both L-amino acids and peptide bonds for cleavage. Furthermore, exopeptidases, which represent the most prominent component of the protease activity in serum, usually act on the first peptide bond of the peptide and require a free N-terminus (Power, et al. (1993), Pharmaceutical Res., 10:1268-1273). In light of this, it is often advantageous to utilize modified versions of subfragment peptides. The modified peptides retain the structural characteristics of the original L-amino acid peptides that confer biological activity with regard to cytokines, but are advantageously not readily susceptible to cleavage by proteases and/or exopeptidases.

**[0029]** Therefore, in specific modes, the peptides of this invention are not subfragment peptides, although their amino acid sequence is derived from the linear sequence of the human cytokine receptors or the corresponding sequences of non-human cytokine receptors and able to inhibit a cytokine receptor's activation (e.g. oligomerization) and more particularly the activation of a human cytokine receptor. Particularly, suitable non-human cytokine receptors sources include mouse, rat, quail and horse. It is thus apparent that multiple systems can provide suitable peptides and derivatives from which the cytokine receptor antagonists of the present invention can be derived.

**[0030]** The term "peptides" as referred to herein therefore includes

cytokine receptor subfragment peptides, D-peptides and other modified forms of the peptides, so long as the modification does not alter ability to modulate cytokine receptor activity. All agonists and antagonists peptides of this invention share the ability to modulate the activity of specific cytokines receptors. Non-limiting examples of modifications include N-terminal acetylation, glycosylation, and biotinylation. Particular modified versions of the subfragment peptides according to the present invention are further described below. Although the peptides of the present invention encompass any peptide derived from the flexible regions of cytokines receptors, preferred peptides of the present invention are chosen so as to be specific to a particular receptor isoform (e.g. VEGFR-2) to ensure that their spatial conformation is complementary to the flexible region that they target. This latter characteristic is obtained by choosing where the peptide will be cut according to the properties afforded by each amino-acid in the remaining sequence (e.g. if the peptide has to follow the specific curve of the domain targeted).

**[0031]** The term "peptides derived from a flexible region" refers herein to peptides of 5 to about 20 amino acids that have been generated to correspond to segments of 5 to 20 contiguous amino acids located anywhere in the flexible regions and that may have been subjected to further modification or functional derivation as described herein. Preferably, the peptides derived from a flexible region is a peptide of at least 7 amino acids.

**[0032]** D-amino acid peptides can have modifications at the N-terminal amino-acid and at the C-terminal amino-acid. The presence of an N-terminal or C-terminal D-amino acid increases the serum stability of a peptide which otherwise contains L-amino acids, because exopeptidases acting on these residues cannot utilize a D-amino acid as a substrate (Powell, et al. (1993)). Cyclic peptides have no free N- or C-termini. Thus, they are not

susceptible to proteolysis by exopeptidases, although they are of course susceptible to endopeptidases, which do not cleave at peptide termini. Thus, the amino acid sequences of the peptides with N-terminal or C-terminal D-amino acids and of the cyclic peptides are usually identical to the sequences of the subfragment peptides to which they correspond, except for the presence of an N-terminal or C-terminal D-amino acid residue, or their circular structure, respectively.

**[0033]** Substitution of unnatural amino acids for natural amino acids in a subsequence of the subfragment of cytokine receptor peptide can also confer resistance to proteolysis. Such a substitution can, for instance, confer resistance to proteolysis by exopeptidases acting on the N-terminus. Such substitutions have been described (Coller, et al. (1993), J. Biol. Chem., 268:20741-20743, incorporated herein by reference) and these substitutions do not affect biological activity. Furthermore, the synthesis of peptides with unnatural amino acids is routine and known in the art (see, for example, Coller, et al. (1993), *supra*).

**[0034]** An other effective approach to confer resistance to peptidases acting on the N-terminal or C-terminal residues of a peptide is to add chemical groups at the peptide termini, such that the modified peptide is no longer a substrate for the peptidase. One such chemical modification is glycosylation of the peptides at either or both termini. Certain chemical modifications, in particular N-terminal glycosylation, have been shown to increase the stability of peptides in human serum [Powell et al. (1993), *supra*]. Other chemical modifications which enhance serum stability include, but are not limited to, the addition of an N-terminal alkyl group, consisting of a lower alkyl of from 1 to 20 carbons, such as an acetyl group, and/or the addition of a C-terminal amide or substituted amide group. In particular the present invention includes modified

peptides consisting of subfragment peptides bearing an N-terminal acetyl group and a C-terminal amide group.

**[0035]** Longer peptide sequences which result from the addition of extra amino acid residues to the peptides of the invention are encompassed in the present invention since they should have the same biological activity (inhibit oligomerization of cytokines) as the peptides described above. While peptides having a substantial number of additional amino acids are not excluded, it will be recognized that some large polypeptides may assume a configuration that masks the effective sequence, thereby preventing binding to cytokines. These derivatives will act as competitive antagonists and are thereby excluded from the invention. Thus, while the present invention encompasses peptides or derivatives having an extension, such longer peptides should be selected as not destroying the modulating activity of the peptide or derivative.

**[0036]** The present invention also encompasses peptides constituted of the sequences of two peptides having separately the property of inhibiting the activation (e.g. oligomerization) of a particular cytokine receptor, but not being contiguous within the flexibility regions. These peptides can also be described as having a sequence corresponding to the particular cytokine receptor with an internal deletion.

**[0037]** In another embodiment of this invention the peptides are reverse-D peptides corresponding to the amino acid sequence of the cytokine. The term "reverse-D peptide" refers herein to peptides containing D-amino acids, arranged in a reverse sequence relative to a peptide containing L-amino acids. Thus, the C-terminal residue of an L-amino acid peptide becomes N-terminal for the D-amino acid peptide, and so forth. For example, the sequence of the reverse-D peptide corresponding to subfragment peptide SEQ ID NO: 1 is:

GVLIIIELNTKEQA. Reverse-D peptides retain the same tertiary conformation, and therefore the same activity, as the L-amino acid peptides, but are more stable to enzymatic degradation *in vitro* and *in vivo*, and thus have greater therapeutic efficacy than the original peptide (Brady and Dodson (1994), Nature, 368: 692-693; Jameson et al. (1994), Nature, 368: 744-746).

**[0038]** As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of an amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. This functional derivative or equivalent may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved (e.g. it acts as a non-competitive inhibitor or agonist of a cytokine receptor). The substituting amino acid generally has chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The term "functional derivatives" is intended to include "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

**[0039]** Thus, the term "variant" refers herein to a protein which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

**[0040]** The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

[0041] While peptides of specific embodiments of the present invention are preferably derived from human cytokines receptors, the invention should not be so limited. Indeed, in view of the significant conservation of flexible regions of these genes throughout evolution, sequences from different species, as discussed above and preferably mammalian species, could be used in the assays of the present invention. For instance, non-limiting examples for the VEGFR protein are the quail, mouse, rat and horse VEGFR protein sequences which show 70%, 82% and 82% similarity, respectively with the human VEGFR protein sequence. Similarly, the IL-1R mouse, rat and horse protein sequences show a 68%, 67% and 77% sequence similarity, respectively. Also, the IL-4R mouse and horse protein sequences show a 48% and 59% sequence similarity, respectively (as calculated by blast™).

[0042] For administration to humans, the prescribing medical professional will ultimately determine the appropriate form and dosage for a given patient, and this can be expected to vary according to the chosen therapeutic regimen (e.g. peptides, variants, mimetics), the response and condition of the patient as well as the severity of the disease.

[0043] Composition within the scope of the present invention should contain the active agent (e.g. peptide) in an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects. Typically, the nucleic acids in accordance with the present invention can be administered to mammals (e.g. humans) in doses ranging from 0.005 to 1 mg per kg of body weight per day of the mammal which is treated. Pharmaceutically acceptable preparations and salts of the active agent are within the scope of the present invention and are well known in the art (Remington's Pharmaceutical Science, 16th Ed., Mack Ed.). For the administration of polypeptides, antagonists, agonists and the like, the amount administered should be chosen so as to avoid adverse side effects. The dosage will be adapted by the clinician in

accordance with conventional factors such as the extent of the disease and different parameters from the patient. Typically, 0.001 to 50 mg/kg/day will be administered to the mammal.

Assays to identify peptides of the present invention

**[0044]** Preferred methods for testing the ability of candidate compounds to inhibit the various cytokine receptors activity are presented herein. It will be understood that the invention is not so limited. Indeed, often assays well known in the art can be used in order to identify non-competitive, extracellular agonists or antagonists of the present invention.

**[0045]** As used herein, "cytokine receptor activity or activation" refers to any detectable biological activity of these proteins. This includes any physiological function attributable to a cytokine receptor such as any standard biochemical measurement of these receptors, conformational changes, phosphorylation status, any downstream effect of the receptor's signaling such as protein phosphorylation, kinase effect or any other feature of the protein that can be measured with techniques known in the art. Measuring the effect of a candidate peptide on its ability to modulate the oligomerization of the receptor is measuring a cytokine receptor's activity according to this invention. Broadly intra- or inter-molecular binding of the receptor in the absence vs the presence of the peptide of the invention is yet another example of a biological activity according to the invention.

**[0046]** The assays of this invention employ either a natural or recombinant cytokine receptor. A cell fraction or cell free screening assays for inhibitors of cytokine receptor activity can use *in situ* purified, or purified recombinant cytokine receptor. Cell-based assays can employ cells which express cytokine receptor naturally, or which contain recombinant cytokine

receptor. In all cases, the biological activity of cytokine receptor can be directly or indirectly measured; thus inhibitors or activators of cytokine receptor activity can be identified. The inhibitors or activators themselves may be further modified by standard combinatorial chemistry techniques to provide improved analogs of the originally identified compounds.

**[0047]** It shall be understood that the *"in vivo"* experimental model can also be used to carry out an *"in vitro"* assay.

*In vitro* assays

**[0048]** In one embodiment, candidate peptides are tested for their ability to activate or inhibit cytokine receptor's ability to modulate cellular proliferation with the incorporated triated thymidine method. In yet other preferred embodiments, candidate peptides are tested for their ability to inhibit a particular cytokine receptor's ability to modulate cellular proliferation, using for example, the assays described in Baker F.L. et al. (1995) Cell Prolif. 28(1):1-15; Cheviron N. et al. ( 1996) Cell Prolif. 29(8):437-46; Hu Z. W. et al. (1999) J: Pharmacol. Exp. Ther. 290(1):28-37; and Elliott K. et al. (1999) Oncogene 18(24):3564-73.

**[0049]** In another preferred embodiment, candidate peptides are tested for their ability to modulate the phosphorylation state of cytokine protein or portion thereof, or an upstream or downstream target protein, using for example an *in vitro* kinase assay. Briefly, a cytokine receptor target molecule (e.g. an immunoprecipitated receptor from a cell line expressing such a molecule), can be incubated with radioactive ATP, e.g., [gamma-$^{32}$P] -ATP, in a buffer containing $MgCl^2$ and $MnCl^2$, e.g., 10 mM $MgCl^2$ and 5 mM $MnCl^2$. Following the incubation, the immunoprecipitated receptor target molecule, can be separated by SDS-polyacrylamide gel electrophoresis under reducing

conditions, transferred to a membrane, e.g., a PVDF membrane, and autoradiographed. The appearance of detectable bands on the autoradiograph indicates that the receptor substrate has been phosphorylated. Phosphoaminoacid analysis of the phosphorylated substrate can also be performed in order to determine which residues on the receptor substrate are phosphorylated. Briefly, the radiophosphorylated protein band can be excised from the SDS gel and subjected to partial acid hydrolysis. The products can then be separated by one-dimensional electrophoresis and analyzed on, for example, a phosphoimager and compared to ninhydrin-stained phosphoaminoacid standards. Assays such as those described in, for example, Tamaskovic R. et al. (1999) Biol. Chem. 380(5):569-78.

**[0050]** In other embodiments, candidate peptides targeting IL-1R are tested with $PGE_2$ levels, IL-6, collagenase expression in chondrocytes and RPE; candidate peptides targeting IGF-1R are tested with Akt in Du145 and PC12; candidate peptides targeting IL-4R are tested with Akt in Thelper and PAEC and with VCAM-1 expression in PAEC.

*In vivo assays*

**[0051]** The assays described above may be used as initial or primary screens to detect promising lead compounds for further development. Lead peptides will be further assessed in additional, different screens. Therefore, this invention also includes secondary cytokine receptors screens which may involve various assays utilizing mammalian cell lines expressing these receptors or other assays.

**[0052]** Tertiary screens may involve the study of the identified inhibitors in animal models for clinical symptoms. Accordingly, it is within the scope of this invention to further use an agent (peptide or peptidomimetic) identified as

described herein in an appropriate animal model such as a rat or a mouse. For example, a peptide can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatment (e.g. treatments of different types of disorders associated with a deregulation or malfunction of a cytokine receptor), as described herein. Preferred such experiments include collagen-induced arthritis in rat, acute septic shock in rat, tumor growth in immunosuppressed mouse, sensitization of the airways in newborn mice and any other known animal model including transgenics.

Assays to identify peptidomimetics

**[0053]** Non-peptidyl compounds generated to replicate the backbone geometry and pharmacophore display (peptidomimetics) of the peptides identified by the methods of the present invention often possess attributes of greater metabolic stability, higher potency, longer duration of action and better bioavailability.

**[0054]** The peptidomimetics compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, Anticancer Drug Des. 12: 145, 1997). Examples

of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) Proc. Natl. Acad. Sci. USA. 90:6909; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al. (1994), J. Med. Chem. 37:2678; Cho et al. (1993) Science 261 :1303; Carrell et al. (1994) Angew. Chem, Int. Ed Engl. 33:2059; and *ibid* 2061; and in Gallop et al. (1994). Med Chem. 37:1233. Libraries of compounds may be presented in solution (e.g.. Houghten (1992) Biotechniques 13:412-421) or on beads (Lam (1991) Nature 354:82-84), chips (Fodor (1993) Nature 364:555-556), bacteria or spores (Ladner USP 5,223,409), plasmids (Cull et al.(1992) Proc Natl Acad Sci USA 89:1865-1869) or on phage (Scott and Smith (1990); Science 249:386-390). Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *supra*; Erb et al. (1994) *supra*; Zuckermann et al. (1994) *supra*; Cho et al. (1993) *supra*; Carrell et al. (1994) *supra*, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

**[0055]** In one embodiment, the peptidomimetics compounds of the present invention are preferably obtained with the following three phase process. 1) Scanning the peptides of the present invention to identify regions of secondary structure necessary for recognition and activity toward the cytokine receptor; 2) use conformationally constrained dipeptide surrogates to refine the backbone geometry and provide organic platforms corresponding to these surrogates; 3) Use the best organic platforms to display organic pharmocophores in libraries of candidates designed to mimic the desired activity of the native peptide. In more details the three phases are as follows.

**[0056]** In phase 1, the peptide leads are scanned and their structure abridged to identify the requirements for their activity. A series of peptide analogs of the original are synthesized. In phase 2, the best peptide analogs

are investigated using the conformationally constrained dipeptide surrogates. Indolizidin-2-one, indolizidin-9-one and quinolizidinone amino acids ($I^2$aa, $I^9$aa and Qaa respectively)) are used as platforms for studying backbone geometry of the best peptide candidates. These platforms are introduced at specific regions of the peptide in order to orient the pharmacophores in different directions. Biological evaluation of these analogs identifies improved leads that mimic the geometric requirements for activity. In phase 3, the platforms from the most active leads are used to display organic surrogates of the pharmacophores responsible for activity of the native peptide. The pharmacophores and scaffolds are combined in a parallel synthesis format.

**[0057]** In summary, based on the disclosure herein, those skilled in the art can develop peptides and peptidomimetics screening assays which are useful for identifying compounds which are useful for inhibiting cytokine receptors. Compounds so identified might also be shown to activate these receptors. The assays of this invention may be developed for low-throughput, high-throughput, or ultra-high throughput screening formats. Of course, assays of the present invention include assays which are amenable to automation.

**[0058]** More specifically, in accordance with one embidoment, the present invention, there is provided a method for identifying a non-competitive peptide which inhibits the oligomerization of a cytokine receptor, the method comprising the steps of selecting a candidate peptide containing from about 7 to about 20 amino acids derived from a flexible region of the receptor, and determining the ability of the peptide to inhibit the oligomerization of the receptor by measuring an activity of the receptor in the presence of a compound known to activate the receptor and in the absence or the presence of the candidate peptide, wherein the non-competitive peptide is selected when the activity of the receptor is measurably lower in the presence of the peptide

as compared to in the absence thereof.

**[0059]** There is also provided a non-competitive extracellular cytokine receptor antagonist wherein the antagonist is a peptide containing from about 7 to about 20 amino-acids derived from a flexible region of the cytokine receptor.

**[0060]** The present invention also provides methods of treating diseases or conditions associated with a abnormal activity of a cytokine receptor comprising administration of a suitable amount of peptide or derivative of the invention.

**[0061]** The present invention also relates to pharmaceutical compositions comprising a modulating amount a or cytokine receptor subfragment peptide or derivative of the present invention, together with a suitable pharmacological carrier.

**[0062]** The terms “inhibiting,” “reducing” or “prevention,” or any variation of these terms, when used in the claims and/or the specification includes any measurable decrease or complete inhibition to achieve a desired result.

**[0063]** The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

**[0064]** It is contemplated that any embodiment discussed in this

specification can be implemented with respect to any method or composition of the invention, and *vice versa*. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

**[0065]** Throughout this application, the term “about” is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

**[0066]** The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

**[0067]** As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

**[0068]** Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0069]** In the appended drawings:

**[0070]** FIG. 1 schematically illustrates the position of VEGFR antagonists on the receptor according to specific embodiments of the present invention;

**[0071]** FIG. 2 graphically illustrates in panel A results of proliferation assay in porcine microvascular endothelial cells in presence of VEGF (2ng/ml) and peptides 2.1, 2.2, 2.3 (10 μM). In panel B are graphically illustrated dose-response of peptides in pulmonary arterial endothelial cells (PAEC) in presence of VEGF (2 ng/ml) and increasing doses of peptides. In panel C is graphically illustrated the effect of intravitreally injected peptides (10 μM [estimated final intraocular concentration]) of the present invention on neovascularization in rat retinas exposed to hyperoxic conditions;

**[0072]** FIG. 3 shows the sequence of the human VEGFR-2 (Flk-1). Boxed or underlined sequences represent the identified flexible region of VEGFR;

**[0073]** FIG. 4 shows the sequence of human Interleukin-1 receptor (IL-1R-alpha). Boxed or underlined sequences represent the identified flexible region of IL-1R-alpha;

**[0074]** FIG. 5 shows the sequence of human Interleukin-1 receptor accessory protein (IL-1RacP). Boxed or underlined sequences represent the identified flexible region of IL-1RacP;

[0075] FIG. 6 shows the sequence of human Insulin-like growth factor I receptor (IGF-1R). Boxed or underlined sequences represent the identified flexible region of; IGF-1R;

[0076] FIG. 7 shows the sequence of the human alpha chain of the Interleukin 4 receptor (IL-4R). Boxed or underlined sequences represent the identified flexible region of; (IL-4R).

[0077] FIG. 8 graphically illustrates results of proliferation assays in carcinome A549 cells in presence of IGF-1 (10ng/ml-Panel A) (1ng/ml-Panel B) and various concentrations of the peptides APG-201, APG-202 and APG-204;

[0078] FIG. 9 graphically illustrates results of proliferation assays in carcinome A549 cells in presence of IL-1 (10ng/ml-Panel A) (1ng/ml-Panel B) and various concentrations of the peptides API-101, API-103 and API-106;

[0079] FIG. 10 graphically illustrates results of proliferation assays in carcinome A549 cells in presence of IL-4 (1ng/ml) and various concentrations of the peptides API-401, API-402, API-403, API-404 and API-405;

[0080] FIG. 11 shows an alignment of the human IL-1R sequence with corresponding mouse, rat and horse sequences;

[0081] FIG. 12 shows an alignment of the human IL-4R sequence with corresponding mouse and horse sequences; and

[0082] FIG. 13 shows an alignment of the human VEGFR2 sequence with corresponding mouse, rat and quail sequences.

## DESCRIPTION OF SPECIFIC EMBODIMENTS

**[0083]** Table 1 presents the localization of flexible regions of various representative members of the cytokine receptors families along with exemplary peptide sequences derived from these regions and chosen for their specificity to the particular member they target. As explained above, many peptides can be derived from the targeted regions of the present invention and the peptides described hereinbelow are only exemplary.

Table 1 : Amino acids involved in the oligomerization and stability of receptors of representative members of various cytokine receptors

| CYTOKINES RECEPTOR TYPES | SPECIFIC RECEPTORS | REGIONS TARGETED | LOCALISATION OF THE SEQUENCE FROM THE STARTING METHIONINE | PEPTIDE SEQUENCES |
|---|---|---|---|---|
| Tyrosine Kinase receptor | VEGFR2 (Flk-1) | Juxtamembranous | Aa 745-770 | AQEKTNLEIIILVG (2.1) SEQ ID NO. 1 |
| | | Ig3-Ig4 | Aa 320-350 | EATVGERVRL(2.2) SEQ ID NO. 2 |
| | | Ig-4 dimerization domain | Aa 350-400 | LPLESNHTLK (2.3) SEQ ID NO. 3 |
| | | Ig-4-Ig-5 | Aa 400-440 | SPVDSYQYGTT; SEQ ID NO. 4<br>VILTNPISKE; SEQ ID NO. 5 |
| | | Ig-5-Ig 6 | Aa 481-565 | NKVGRGERVI; SEQ ID NO. 6<br>MPPTEQESV SEQ ID NO. 7 |
| | | Ig-6-Ig-7 | Aa 640-685 | RKTKKRHCV; SEQ ID NO. 8<br>TVLERVAPT; SEQ ID NO. 9<br>TSIGESIEV SEQ ID NO. 10 |
| | IGF-1R | On chain $\alpha$: | | |
| | | Juxtamembranous | Aa 725-740 | SIFVPRPERK SEQ ID NO. 11;<br>NFLHNSIFV SEQ ID NO. 12; |
| | | Cyst rich domain-L2 | Aa 320-335 | EGPCPKVCE SEQ ID NO. 13; |
| | | L2-FbnIII-1 | Aa 487-527 | ESDVLHFTST SEQ ID NO. 14; |
| | | FbnIII-1-FbnIII2a | Aa 595-620 | RTNASVPSI SEQ ID NO. 15; |
| | | FbnIII-2a-Insert domain | Aa 660-690 | IRKYADGTI SEQ ID NO. 16; |
| | | On chain $\beta$: | | |
| | | Insert domain-FbnIII2b | Aa 780-799 | ENFIHLIIA SEQ ID NO. 17;<br>AKTGYENFIH SEQ ID NO. 18; |
| | | FbnIII2b-FbnIII3 | Aa 820-840 | KERTVISNLR SEQ ID NO. 19; |
| | | Juxtamembranous | Aa 917-947 | FVFARTMPA SEQ ID NO. 30; |
| | EGFR | Juxtamembranous | Aa 640-650 | NGPKIPSIAT SEQ ID NO. 31; |

| | | | | |
|---|---|---|---|---|
| | | Loop L2-S2 (flexible) | Aa 495-515 | ATGQVCHAL SEQ ID NO. 32; |
| | | Loop S1-L2 (Hinge) | Aa 335-345 | RKVCNGIGIGE SEQ ID NO. 33; |
| Type I: Chain γc | IL-4R | Juxtamembranous | Aa 210-240 | WHNSYREPF SEQ ID NO. 34; YREPFEQHLL SEQ ID NO. 35 |
| | | Hinge zone D2 | Aa 125-216 | SDTLLLTWS SEQ ID NO. 36; IYNVTYLE SEQ ID NO. 37; IAASTLKSGIS SEQ ID NO. 38; |
| | | Loop D1-D2 | Aa 112-125 | KPSEHVKPR SEQ ID NO. 39; |
| Single chain | GHR | Juxtamembranous flexible region (D1-D2) | Aa 250-270 Aa 160-240 | FTCEEDFYFPW SEQ ID NO. 40; SVDEIVQPD SEQ ID NO. 41; MDPIDTTSVPVY SEQ ID NO. 42; |
| IL-1R | IL-1R | Juxtamembranous | Aa 320-341 | IDAAYIQLIYPV; SEQ ID NO. 43 LIYPVTNFQKHM SEQ ID NO. 44 |
| | | Between Ig-like domain 2 and 3 (Hinge) | Aa 209-240 | LEENKPTRPV; SEQ ID NO. 45 NKPTRPVIVS SEQ ID NO. 46 |
| | | Ig-like 2 loop e2-f2 (pas int.ligand) | Aa 181-200 | VAEKHRGNYT; SEQ ID NO. 47 WNGSVIDED SEQ ID NO. 48 |
| | IL-1RacP | Juxtamembranous | Aa 330-370 | VPAPRYTVEL SEQ ID NO. 49; APRYTVELA SEQ ID NO. 50; |
| | | Hinge regions: | | |
| | | Loop Ig-1-2: | Aa 115-160 | VQKDSCFNSPM; SEQ ID NO. 51 MKLPVHKLY SEQ ID NO. 52 |
| | | Loop Ig-2-3 | Aa 170-266 | VGSPKNAVPPV SEQ ID NO. 53; VTYPENGRTF SEQ ID NO. 54; IHSPNDHVVY SEQ ID NO. 55; |
| | | dimerization region | Aa 200-215; 275-295; 300-315 | LISNNGNYT SEQ ID NO. 56; VWWTIDGKKPD SEQ ID NO. 57; WTIDGKKPDDI SEQ ID NO. 58; HSRTEDETRTQ SEQ ID NO. 59 |

[0084] Cytokines receptors modulators according to specific embodiments of the present invention will now be described as well as the procedure to identify them and to test their efficiency *in vitro* and/or *in vivo* by the following non-limiting examples.

## EXAMPLE 1

## VEGFR

Identification of VEGFR2 antagonists

[0085] VEGF is a proliferating agent for endothelial cells. Its receptor (VEGFR) is present at the plasma membrane of endothelial cells as a monomer and its homodimerization is necessary for generating autophosphorylation via its intrinsic tyrosine kinase domain.

[0086] The method of identifying VEGFR antagonists of the present invention is based on the localization of extracellular flexible regions including regions between domains and juxtamembranous regions of the receptor that are important for the appropriate conformation and oligomerization of the subunits of the receptor and its resulting activation. These regions were established based on crystal structure data provided by crystallography. The antagonists able to bind to these regions block the signal transduction by interfering with the oligomerization. The regions so identified appear in green in Figure 3. One of those regions is located under the IG-like 3 domain where ligand binding is located, namely between residues 320 and 350. The ligand binding location also appears in Figure 1. A second region was identified in the oligomerization domain of two subunits of Ig-like 4, namely between residues 350 and 400. A third region was identified located at the juncture of the receptor with the cellular membrane, namely between residues 745 and 770. This region is important for the dimer stability. These regions do not interfere

with the ligand binding so that any antagonist (peptide, small molecule) targeting these regions is not a competitor for the ligand binding sites (non-competitive antagonist) and prevents or limits the oligomerization required for the autophosphorylation of the receptor. Three D-peptides of up to 12 amino-acids (designated 2.1, 2.2 and 2.3) were derived from the amino-acid sequence of these regions and tested as antagonists. As mentioned earlier, D-peptides are preferred over subfragment peptides (of course subfragments could also be rendered protease resistant by well known means) because they are less likely degradable by various proteases. These particular peptides were selected among all those that could have been derived from the identified flexible regions of interest because of their specificity to VEGFR-flk-1: sequences alignments were performed with other receptors from VEGFR's family (PDGFR, Flt-1) showing the specificity of the selected three peptides. Of course, such alignments enable a selection of other specific peptides or alternatively of more general antagonists. It should be understood that the principles related to positioning discussed herein in relation to VEGFR can be applied to other types of cytokine receptors sharing similar morphologies.

**[0087]** The location of the three peptides appear in Figure 1, the ligand binding region appears in red, the oligomerization domain *per se* appears in green and the tyrosine kinase domain appears in purple.

**[0088]** In Figure 3, the domains of the VEGFR isoform VEGFR-2 are identified with arrows pointing at the start of each domain. The regions where antagonists of the present invention may bind to prevent the oligomerization and/or activation of the receptor are boxed or underlined. The underlined sequences denote the regions between domains while the boxed sequences denote the juxtamembranous regions. The regions from where peptides 2.1, 2.2 and 2.3 are derived are identified in *italic* and are underlinded. The

sequences that the peptides target according to the invention appear underlined and boxed.

Characterization of peptides *in vitro*

**[0089]** To determine the efficient and non cytotoxic concentration of VEGF to use in the assay, a dose-response curve of VEGF was generated in two types of cells, namely microvascular endothelial cells and pulmonary artery endothelial cells (PAEC) that had been transfected with the Flk-1 gene. The proliferation was then measured in those two types of cells in the presence of peptides 2.1, 2.2 and 2.3 and of VEGF (2ng/ml) pursuant to the incorporated tritiated thymidine method. The cells were preincubated at 37°C with the different peptides at different concentrations. They were incubated with VEGF (2ng/ml) for 24 hours. The cells were contacted with $^3$H-Thymidine for 24 hours, washed and lysed. The radioactivity was measured with a scintillation counter.

**[0090]** As may be seen in panels A and B of Figure 2, the peptides 2.1, and 2.2 completely abrogated VEGF induced proliferation in microvascular endothelial cells, and in PAEC with an $EC_{50}$ of 9 $\mu$M, respectively. In addition, using these PAEC transfected with the cDNAs for either of the VEGFR isoforms Flk-1 and Flt, the selectivity of the peptides was demonstrated as they were shown to be ineffective in modulating biological functions in the VEGFR Flt isoform -containing cells (data not shown).

Characterization of peptides *in vivo*

Ischemic retinopathy model

**[0091]** The efficiency of the selected peptides was verified *in vivo* in a ischemic retinopathy model, a phenomena highly dependant on VEGF activation. Rat pups were exposed to 80% $O_2$ followed by a period of normoxia (21% $O_2$). The peptides were injected at a final concentration of 10$\mu$M in the

vitreous body. The retinas were then retrieved, colored with the ADPase method and mounted on slides. Photographs of the retinas were taken with a microscope linked to a computer and the vascular density was evaluated with the Image prosoftware. As illustrated in Figure 2, panel C, the results of this experiment demonstrated that all peptides tested prevented induced neovascularization *in vivo*. Peptide 2.2 was shown to be the most effective inhibitor of neovascularization. Specific peptides of the present invention were shown to prevent effects generated by activation of Flk-1 with VEGF by interfering with flexible regions of Flk-1 receptor.

## EXAMPLE 2

### Insulin-like growth factor-1 receptor (IGF-1R)

**[0092]** IGF-1 is a small peptide and a member of a family of insulin related peptides. It consists of 70 amino acids and has structural similarity with insulin. IGF-1 is secreted by many tissues (cartilage, bone, epithelium, endothelium) but mostly by the liver to act on other tissues in an endocrine fashion. It exerts its actions by binding to IGF-1R upon which it sends a mitogenic signal. It can also protect cells from apoptosis, promote proliferation, regulate cell adhesion and motility and differentiation. The receptor itself is expressed in most cell types except in the hepatocytes. Because of its growth inducing functions, IGF-1R is also very much involved in malignant transformation or differentiation in various types of cancer such as glioblastomas, neuroblastomas, prostate, breast and ovarian cancer.

**[0093]** IGF-1 plays a critical role in cell growth, survival and metastatic differentiation. IGF-1R is a transmembrane tyrosine kinase protein, which is widely expressed. Increased IGF-1 R expression is observed in a number of tumour types, and epidemiological data implicates it in cancers such

as those of the prostate and breast. Recent progress has been made on its 3-dimensional structure.

Design of peptides for IGF-1

**[0094]** The approach described in Example 1 is used to generate antagonists to IGF-1R. The precise localization of these regions is described in Table 1 above along with exemplary sequences of subfragment peptides or modified peptides targeting one of these regions and presenting specificity to IGF-1R. Three D-peptides (designated APG201, APG202 and APG204) were then derived from the amino-acid sequence of these regions to act as antagonists. The sequences of these peptides antagonists are as follows: APG-201 SLFVPRPERK; APG-202 ESDVLHFTST; APG-204 LRKYADGTL. They generally correspond to the subfragment peptides having sequences SEQ ID NOs: 11, 14 and 16, respectively, except where the subfragment peptide contained an isoleucine. Similarly, In that case, this amino-acid was replaced by leucine in the synthesized peptide for economic reasons.

Characterization of peptides *in vitro* and *in vivo*

**[0095]** The affinity is determined using binding studies on cells expressing and overexpressing IGF-1R. The selectivity is tested by performing bioassays on cells expressing receptors from the same family as IGF-1R and the specificity is tested against receptors of another family of cytokine.

**[0096]** The proliferation of IGF-1 was measured in A549 carcinoma cells in the presence of peptides APG201; APG202 and APG204 and of IGF-1 (10ng/ml-Panel A) and (1ng/ml-Panel B) pursuant to the incorporated tritiated thymidine method. The cells were preincubated at 37°C with the different peptides at different concentrations, namely $10^{-7}$, $10^{-6}$ and $10^{-5}$M. They were then incubated with IGF-1 (10ng/ml or 1 ng/ml) for 24 hours. The cells were

then contacted with $^3$H-Thymidine for 24 hours, washed and lysed. The radioactivity was then measured with a scintillation counter.

[0097] As may be seen in panels A and B of Figure 8, the peptides completely abrogated IGF-1 induced proliferation in A549 carcinoma cells with an $EC_{50}$ of $10^{-8}$M for APG-202 and 204; and of $10^{-6}$M for APG-201.

[0098] Further *in vitro* testing of the antagonists are conducted as described in Table 2.

[0099] *In vivo* experiments are described in Table 5.

**Table 2. *In vitro* bioassays for IGF-1R antagonist screening**

| Cells | Type | Bioassay | Method |
|---|---|---|---|
| Du145 | Prostate cancer cell line | - Proliferation<br>- Akt phosphorylation | $^3$H-Thymidine incorporation<br>Western Blot |
| PC12 | Pheochromo-cytoma cell line | Same as above | Same as above |

## EXAMPLE 3

### Interleukin 4 (IL-4)

[00100] IL-4 is a key cytokine involved in the development of allergic inflammation and allergy. It is generated early on in the process of inflammation in asthma. In allergy it is associated with the production of IgE immunoglobulins by B lymphocytes and will also up-regulate the expression of the IgE receptor on cell surface of B-lymphocytes, basophils and mast cells. In asthma it induces the expression of vascular cell adhesion molecule (VCAM-1) on vascular endothelium. This effect leads to direct migration chemotaxis of T lymphocytes, monocytes, basophils and eosinophils to the inflammatory site on

pulmonary vascular endothelial cells. IL-4 inhibits eosinophil apoptosis and promotes eosinophilic inflammation by augmenting their presence in part by increasing expression of eotaxin. Another essential biological effect of IL-4 is Th2 differentiation and proliferation; in this process IL-4 diminishes T lymphocyte apoptosis. The Il-4 receptor is a cell-surface protein consisting of an $\alpha$ subunit coupled to a $\gamma$ subunit for signal transduction; its activation requires oligomerization.

**[00101]** Although IL-4R and IL-13R share a similar IL-4R$\alpha$ chain, the two receptors exhibit distinct functions; moreover, the main receptor present on TH2 cells is that of IL-4, which for the most part consists of the IL-4R$\alpha$ and IL-4$\gamma$c chains. Nevertheless, the identification of modulators of IL-4R activity. Derived from the IL-4R$\alpha$ are expected to also modulate IL-13R activity.

Design of peptides for IL-4R

**[00102]** The approach described in Example 1 is used to generate antagonists to IL-4R. The precise localization of these regions is described in Table 1 above along with exemplary sequences of subfragment peptides or modified peptides targeting one of these regions and presenting specificity to IL-4R

Characterization of peptides *in vitro* and *in vivo*

**[00103]** The affinity is determined using binding studies on cells expressing and overexpressing IL-4R. The selectivity is tested by performing bioassays on cells expressing receptors from the same family as IL-4R and the specificity is tested against receptors of another family of cytokine.

**[00104]** The proliferation of IL-4 was measured in A549 carcinoma

cells in the presence of peptides API-401, API-402, API-403, API-404 and API-405 and of IL-4 (1ng/ml) pursuant to the incorporated tritiated thymidine method. The cells were preincubated at 37°C with the different peptides. They were then incubated with IL-4 (1 ng/ml) for 24 hours. The cells were then contacted with $^3$H-Thymidine for 24 hours, washed and lysed. The radioactivity was then measured with a scintillation counter. The sequences of peptides antagonists used are as follows: API-401 YREPFEQHLL, API-402 SDTLLLTWS; API-403 LYNVTYLE; API-404 LAASTLKSGLS; and API-405 KPSEHVKPR. They generally correspond to the subfragment peptides having sequences SEQ ID NOs: 35, 36, 37, 38 and 39, respectively except where the subfragment peptide contained an isoleucine. In that case, this amino-acid was replaced by leucine in the synthesized peptide as mentioned previously.

**[00105]** As may be seen in Figure 10, four out of five peptides prevented IL-4 from stopping proliferation in A549 carcinoma cells.

**[00106]** Further *In vitro* testing of the antagonists is conducted as described in Table 3. *In vivo* experiments are described in Table 5.

**Table 3. *In vitro* bioassays for IL-4R antagonist screening**

| Cells | Type | Bioassay | Method |
|---|---|---|---|
| T helper | T helper cells | - Proliferation<br>- Akt phosphorylation | $^3$H-Thymidine incorporation<br>Western Blot |
| PAEC | Human pulmonary artery endothelial cells | VCAM-1 expression | Western blot |

## EXAMPLE 4

### Interleukin-1

**[00107]** Interleukin-1 (IL-1) plays a primary upstream role in the

regulation of inflammation by stimulating generation of other inflammatory mediators and by enhancing the process of inflammation directly. Along with TNF, IL-1 is considered as a prototype for inflammatory cytokines. The effects of IL-1 are not limited to inflammation and this cytokine plays a role in bone formation and remodeling, insulin secretion and fever induction. IL-1 is also a major player in acute and chronic inflammation (e.g. septic shock, inflammatory bowel diseases, osteoarthritis, or rhumatoid arthritis), Alzheimer's disease and a number of autoimmune diseases. Monocytes are predominant sources of IL-1 but many other cell types express the protein: non-limiting examples include fibroblasts, endothelial cells, smooth muscle cells, osteoclasts, astrocytes, epithelial cells T-cells, B-cells and numerous cancer cells.

**[00108]** The interleukin-1 family of proteins consists of distinct but structurally related molecules: IL-1$\alpha$, IL-1$\beta$ and IL-18 which elicit a biologic response and IL-1ra, a naturally produced receptor antagonist. IL-1$\alpha$ is the predominant form in mice, IL-1$\beta$ is predominant in human but both exert their effect through the same receptor. In addition, IL-1 induces the production of other inflammatory mediators like IL-6 and prostaglandin $PGE_2$ (induces COX-2 and PGE synthase expression) and induces proliferation and activation of numerous cell types.

**[00109]** As a major pro-inflammatory cytokine, IL-1 is a potentially powerful target for therapeutic interventions in diseases associated with articular cartilage injury such as in arthritis. Osteoarthritis and rhumatoid arthritis are only second to heart diseases for causing work disabilities in USA and their prevalence increase dramatically with age. Approximately 60 millions of American >40 years of age are at risk. In 1997, direct medical and disability costs for arthritis were approximately $75B (US). Other important disorders for which IL-1 contributes significantly include ulcerative colitis and Crohn's

disease, which are also major causes of absenteeism in USA, and other types of auto-immune diseases.

**[00110]** Two distinct receptors of IL-1 have been cloned and characterized: IL-1R which generates the biological effects of IL-1, and IL-1RII which is a natural antagonist. In addition, a receptor accessory protein (IL-1RAcP), which is the putative signal-transducing subunit of the receptor complex has been identified. IL-1R type I is found mainly on T cells, keratinocytes, fibroblasts, chondrocytes, synoviocytes and epithelial cells. In order to generate a biological effect, IL-1R has to bind to IL-1 and subsequently to IL-1RacP which is necessary for signal transduction. The extracellular portion of IL-1R contains 3 Ig-like domains that bind IL-1. Of note, according to studies involving antibodies directed against extracellular portions of IL-1RacP, the latter does not interact with the cytokine and could therefore also be an excellent target for non-competitive peptidomimetic design.

Design of peptides for IL-1R

**[00111]** The regions of the IL-1 receptor complex which were targeted are the third domain of IL-1R containing a flexible region and interacts with the accessory protein but not with the ligand. The equivalent domain on IL-1RacP, is the juxtamembranous regions of IL-1R and IL-1AcP and the regions between the second and third extracellular domains of IL-1RacP. The precise localization of these regions is described in Table 1 above along with exemplary sequences of subfragment peptides or modified peptides targeting one of these regions and presenting specificity to IL-1R.

Characterization of peptides *in vitro* and *in vivo*

**[00112]** The affinity of the subfragment peptides or derivative is determined using binding studies on cells expressing or overexpressing IL-1R.

The selectivity is tested by performing bioassays on cells expressing receptors from the same family as IL-1R (e.g. IL-18R) and the specificity is tested against receptors of another family of cytokine.

**[00113]** The proliferation effect of IL-1 was measured in A549 carcinoma cells in the presence of peptides API101; API103 and API106 and of IL-1 (10ng/ml-Panel A) and (1ng/ml-Panel B) pursuant to the incorporated tritiated thymidine method. The cells were preincubated at 37°C with the different peptides at different concentrations, namely $10^{-6}$, $10^{-5}$ and $10^{-4}$M. They were then incubated with IL-1 (10ng/ml or 1 ng/ml) for 24 hours. The cells were then contacted with $^3$H-Thymidine for 24 hours, washed and lysed. The radioactivity was then measured with a scintillation counter. The sequences of the peptides antagonists used are as follows: API-101 APRYTVELA, API-103 MKLPVHKLY; and API-106 VGSPKNAVPPV. They generally correspond to the subfragment peptides having sequences SEQ ID NO: 50, NO: 52 and NO: 53, respectively except where the subfragment peptide contained an isoleucine. In that case, this isoleucine was replaced by a conservative leucine in the synthesized peptide for economic reasons.

**[00114]** As may be seen in panels A and B of Figure 9, the peptides completely abrogated IL-1 induced proliferation in A549 carcinoma cells with an $EC_{50}$ of $10^{-6}$M for API-101 and 103; and of $10^{-5}$M for API-106.

**[00115]** The goal of the next experiment was to verify if the identified peptides can reverse the physiological actions of the natural cytokine *in vivo* either by injecting them through the jugular or directly in the stomach (to verify the stability of the peptide through the digestive tractus). 300 g Sprague-Dawley rats were anesthetized with isoflurane (2.5-4%). IL-1β was injected through the jugular. Blood was taken from the carotid for further analyses

before and after (10 minutes) every injection. Peptides were then injected either directly in the stomach with a catheter or in the jugular at the concentration desired. Arterial blood pressure and other physiological characteristics were monitered at all time.

**[00116]** A severe hypotension induced by IL-1β was observed when administrated to the rats by either ways mentionned above. The following peptides constitute examples of antagonists that were able to prevent hypotension:

**[00117]** API-101.10 (target : juxtamembranous portion of the accessory protein of IL-1R, derivative of API-101) :

1)When administrated by jugular injection after IL-1β injection (5ug/kg) it prevented hypotension by 95% at a concentration of $10^{-8}$M. This demonstrated that the peptide has an hypotensor effect *in vivo* in animals by reversing the effect of IL-1β (data not shown).

2) When administrated directly into the stomach, the peptide at a concentration of $10^{-5}$M, reduced IL-1β induced hypotension by 60 %. This result demonstrated that oral administration of the 101.10 peptide still maintained a major effect on IL-1β induced hypotension. (Data not shown)

**[00118]** In another experiment, vasomotricity variation of piglets pial vessels was studied to further evaluate the particular effect of cytokine receptor subfragments on the vasodilatator effect of IL-1β. Brains were dissected from Yorkshire piglets. Slices of brain exposing the pial vessels were pinned to a wax base of a 20 ml bath containing Krebs buffer (pH 7,4) equilibrated with

$95\%O_2$-$5\%$ $CO_2$ and maintained at 37°C. Microvessels were visualized and recorded using a video camera mounted on a dissecting microscope. Vascular diameter was measured using a digital image analyzer and the images were recorded before and after topical application of constricting agent U46619 at $10^{-7}$M. After stabilization of the vasomotricity, IL-1β was added until stabilization of vasodilatation. Peptides were then injected at different concentrations from $10^{-10}$ to $10^{-5}$M. Reversal of vasodilatation (i.e. vasoconstriction) was visualized and measured as previously mentionned. IL-1β induced vasodilatation in the microvasculature of the piglet brain was observed. Examples of the inhibitory activity of cytokine subfragment peptides are given below:

1)API-101 and 101.10 (Juxtamembranous part of accessory protein) could prevent the vasodilation induced by IL-1β (75 ng/ml) with an $IC_{50}$ of 182 nM (API-101) and 10.8 nM (API-101.10). The range of concentrations of the peptide administered was from $10^{-10}$ to $10^{-5}$M (data not shown)

2)API-108 (hinge Ig-3 region of accessory protein) could prevent vasodilatation with an $IC_{50}$ of 1.9 nM (data not shown). The range of concentrations of the peptide administered was from $10^{-10}$ to $10^{-5}$M.

**[00119]** These results demonstrate that targeting of two flexible regions of one component of the receptor we could prevent IL-1β activity at a very low $IC_{50}$ and therefore with a very high efficiency.

**[00120]** Another way of assessing the effect of cytokine receptor subfragments on IL-1R activity *in vivo* is by measuring $PGE_2$ levels in rat blood serum . Rat blood samples were collected from *in vivo* experiments (e.g. Protocol for IL-1 induced hypotension) and centrifuged at maximum speed for

15 minutes. The serum was then passed through a Waters column in order to isolate the lipidic part. Samples were evaporated and $PGE_2$ quantities were determined with an RIA assay using a commercial kit (Cederlane).

**[00121]** If the cytokine receptor subfragment peptides can prevent hypotention *in vivo* they should be able to prevent also the synthesis of $PGE_2$. The prostaglandin was therefore measured in serum of rats used for experiments mentioned above (e.g. Arterial Blood Pressure variation measurement) . An example of results obtained with a particular cytokine receptor subfragment peptide is described below:

1) API-101.10 could prevent $PGE_2$ synthesis *in vivo* by 80% when the peptide was injected in the jugular. The same results were obtained when the peptide was injected directly in the stomach (data not shown).

**[00122]** These experiments demonstrate that the identified peptides derived from different flexible regions of a cytokine receptor (in this particular example, receptor IL-1R/IL-1RacP) are efficient and very potent *in vitro* and *an vivo* at reversing various biological effects of IL-1$\beta$.

**[00123]** From these experiments the efficiency and specificity of the method used to select particular cytokine subfragment peptides to modulate cytokine receptor activity is clearly demonstrated. Furthermore, the particular experiments presented above (with the IL-1R/IL-1RacP receptors) serves as a complete example of how one can select a particular cytokine receptor subfragment peptide (derivitize and /or protect it if desired), test its modulating activity *in vitro* and than its efficiency and potency *in vivo.* It also demonstrate that the modulating activities demonstrated *in vitro* are translatable to the *in*

*vivo* situation.

[00124] The stability and selectivity of the peptides *in vitro* and *in vivo* is further verified with the tests described in Table 4, and Table 5 below, respectively.

**Table 4. *In vitro* bioassays for IL-1R antagonist screening**

| Cells | Type | Bioassay | Method |
|---|---|---|---|
| Chondrocytes | Human chondrocytes | - $PGE_2$ levels<br>- IL-6<br>- Proliferation<br>- Collagenase expression | RIA kit<br>RIA kit<br>$^3$H-Thymidine incorporation<br>Western Blot |
| RPE | Human retinal pigment epithelial cells | Same as above | Same as above |
| Thymocytes | EL4 - Mouse thymocytes -High IL1R expression | Proliferation | $^3$H-Thymidine incorporation |
| Fibroblasts | Human F7100 | Proliferation | $^3$H-Thymidine incorporation |

[00125] Table 5 summarizes the nature of the *in vivo* experiments performed with various peptides of the present invention. They are presented in more details below.

Table 5. *In vivo* experiments to assess efficacy and specificity of antagonists against IL-1R, IGF-1R and IL-4R

| Target | Animal model | Method | Treatment | Parameters |
|---|---|---|---|---|
| **IL-1R** | Collagen-induced arthritis in rat | s.c. injections of type II collagen in incomplete Freund's adjuvant | Following onset of arthritis, continuous delivery of the drug via osmotic pump | Destruction of cartilage assessed by histological staining and digital imaging |
| | Arterial blood pressure variation measurement in rats | Injection of IL-1b in jgular | 10 minutes following IL-1b, injection of peptide antagonist in jugular or stomach. | Blood pressure variation measurements |
| | Vasomotricity experiment on piglet pial vessels | Topical application of U46619 agent as vasoconstrictor than, IL-1b as a vasodilatator | Following U46619 induced vasodilatation, application of peptide antagonist in microvessels | Vascular diameters |
| | $PGE_2$ levels in rat blood serum | Injection of IL-1b in jugular | injection of peptide antagonist in jugular or stomach and measurement of $PGE_2$ levels by RIA kit | $PGE_2$ levels |
| | Acute septic shock in rat | LPS-induced septic shock | Preceding i.v. bolus of LPS the animal will receive an i.v. bolus of the antagonist | Blood pressure, body temperature and cardiac rhythm will be monitored during the whole experiment (60 min) |
| **IGF-IR** | Tumor growth in immunosuppressed mouse (nude mouse) | s.c. injection of tumoral cell line | Continuous delivery of the antagonist with osmotic pump after latency to obtain solid tumor | Tumor size monitoring |
| **IL-4R** | Sensitization of the airways in newborn mice | Exposure of the animals ovalbumin (i.p. injection and aerosolized) | i.p. injection of receptor antagonist | IgE and TNF-$\gamma$ dosage |

Acute septic shock in rats

**[00126]** The efficiency of the peptides is also verified with the acute septic shock in Sprague-Dawley rat. Sprague-Dawley (160-180 gm) rats (Charles River) are anesthesed with a solution 9 :1 xelazine/ketamine at a concentration of 1 mg/Kg. A tracheotomy is performed so as to maintain ventilation with a tube linked to a respirator. A cannula is inserted into the right carotide artery to enable monitoring of the systemic arterial with a Stratham pressure transducer linked to a multichannel Gould apparatus. The right jugular vein is cannuled to enable drug administration. The animal is placed under radial heat to maintain a constant normal temperature. The septic shock is obtained by systemic injection of a lipopolysaccharide bolus (LPS) (1 mg/kg : Sigma). A decrease of about 30 mm Hg is observed after ~5 minutes.

Collagen-induced arthritis protocol in Lewis rat

**[00127]** Type II Collagen (CII) that has been isolated and purified from bovine articulary is obtained from Sigma. CII (2 mg/ml) is dissolved over night at 4°C with agitation in 0,01 M acetic acid. The solution is then emulsified in an incomplete Freund's adjuvant (CII : ICFA, Difco Laboratories, Detroit, MI). Lewis female rats (Charles River) of 140-180 gm and of 8 week old are immunised with 0,5 ml of the emulsion (0,5 mg CII) with many intradermal injections in the back and one or two injections in the tail base. The animals are then reinjected 7 days later in the tail base with 0,2 ml (0,2 mg CII) so as to obtain an acute inflammatory reaction. A different time points during the experiment (1 to 24 days) animals are sacrificed and knuckle joints samples are taken to be fixed and coated so as to enable cryosections of 6-7 µm. A double coloration of Goldner and toluidine blue is performed on slides to measure the importance of the articular inflammation. Digitalised images are taken and analysed with the Image Pro Plus™ 4.1 software.

Tumor growth in immunosuppressed mouse (nude mouse)

**[00128]** The colon Colo 205™ carcinoma cell line is obtained from the American Type Culture Collection (ATCC : Rockville, MD). Cells are maintained in a RPMI-1640 culture and grown in 100 mm Petri at 37°C in a humidified atmosphere controlled to maintain 5% CO2 and 95% air. The medium is supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

**[00129]** $2{,}5 \times 10^6$ carcinoma colon Colo 205™ cells in 100 µl de PBS are injected subcutaneously in the back (needle 25 G : BD, NJ) in 6 weeks old immunodeficient female mice (Balb/c, nu/nu : Charles River). Treatment begins 5 days after injection of tumorous cells measuring ~0,5 x 0,5 cm. the tumour volume is measured every two days according to the following formula: lenght x width x hight, with a vernier caliper. 14 days after the beginning of treatments, animals are sacrificed and tumours are sampled to be weighted and measured in volume. Specimens are then fixed in a 10% formalin buffer for 24H and then transferred in 70% ethanol. Tumours are then coated with paraffin and sections are cut for immunohistochemistry purposes. The general morphology is evaluated with a hematoxyline / eosin coloration.

**[0100]** Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims. In particular, although the flexible regions of all cytokines have not all been described herein nor have all peptidic extracellular non competitive modulators encompassed by the present invention targeting these regions have been described, in light of the procedure described above for screening peptides and identifying peptides of the present invention, a person of ordinary skill in the art would be able to rapidly develop peptidic

modulators of cytokine receptor by selecting peptides of 5 to 20 amino acid derived from known flexible regions of cytokines.

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